Linkage between Cariogenic Streptococcus Mutans and Atherosclerotic Plaques of Cardiovascular Disease Patients in Iraqi Population

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Abstract

This study was done to identify the correlation between cariogenic S. mutans and CVD patients of Iraqi population.

Materials and Method: Eighty six cardiovascular Iraqi patients between (24-70) years old were investigated for their association with cariogenic S. mutans. Pathological samples of vascular and heart valve biopsies and atherosclerotic plaques from the catheter tips of the diagnostic and therapeutic catheters of CVD patients were analyzed with molecular PCR technique with two types of primers, 16s universal primer and S. mutans serotype-specific primer sets genotyping was performed to determine the linkage between cariogenic S. mutans and CVD patients in Iraqi population.

Results: DNA samples of cariogenic S. mutans was specified in 60 / 86(69.7%) of the enrolled cardiovascular disease patients, distributed according to the gender between 42/60 (70 %) in males and 18/60 (30%) in females and according to the age between 33/60(55 %) of the positive patients were at 24-60 years and 27/60 (45 %) of the positive patients were > 60 years old.

Keywords: *Cardiovascular disease, Biofilm, Cariogenic Streptococcus mutans, PCR; Universal 16S ribosomal-RNA and Serotype-specific primers genotyping.*

Introduction

Cardiovascular diseases are the major etiological causes of both weakness and mortality in numerous developing and developed countries, It is expected that during the next time, heart diseases will be the direct cause of fatality in human being [(1)]., Cardiovascular diseases are the reason of approximately 40% of mortality in Europe each year and they killed over 3.9 million people, the bulk of deaths were due to heart disease and stroke [(2)]. Cardiovascular diseases refer to many different illnesses that attack the heart muscle, blood vessels and valves due to biofilms formation, atherosclerotic plaques, very broad etiological agents involved in these diseases including factors such as age, sex, and diet [(1)] . Biofilm are considered as accumulation of microbial masses that adheres to solid surfaces in dental plaques or intravenous catheter or as synergistic with polysaccharide matrix in extracellular. About 65% of infections in human are because of formation of microbial biofilms [(3)]. The biofilms are created by original adherence of bacteria to a solid surfaces, progress of a multi-dimensional intricate structure and disinterest to development in other place. The greatest example of biofilm development is dental plaques which lead to dental caries, periodontitis and other associated systemic disorders. [(5)]. Despite of the high diversity of the human oral microbiota, and its close contact with the circulatory system, various bacteria are implicated in biofilm formation, Streptococcus mutans is the fundamental pathogen in oral cavity which is causal factor of dental plaques and dental caries, considered the most common diseases throught the world [(6 , 7)]. This pathogen which pass through the bloodstream may results in infective endocarditis [(8-9)]. Many previously studies recorded that periodontopathic bacterial species, as well as several Streptococcal species, were specified in cardiovascular specimens like heart valve and atheromatous plaque, and S. mutans was the higher counts that diagnosed [(4-10)] .

Materials and Method

The present study was performed during the period of July 2018 to September 2019, collection of specimens was in Cardiology Unit of AL-Hussein Educational Hospital in Kerbala City for (86) cardiovascular disease patents distributed into (42 males and 18 females), aged between (24 to 70) years old who received endarterectomies, catheter-based atherectomy, or similar procedures because of various manifestations of ischemia for heart valve and blood vessels and atherosclerotic patients. Also a pool of (23) diagnostic catheterization tissue specimens were from 15 males and 8 females that obtained as a control group.

Subjects and Collection of Specimens

After clinical diagnosis of CVD patients, the vascular and atheromatous plaque biopsy samples from the tips of the therapeutic and diagnostic catheters of CVD patients and control group were achieved with highly sterile conditions, each endarterectomy specimen was immediately transferred into1.5 ul polypropylene microcentrifuge tube contained 500 ul of 0.9% sterile normal saline solution, and rapidly transferred and subjected to the laboratory for molecular investigation.

Isolation of DNA

DNA Extraction from all of the vascular and atheromatous plaque biopsies was carried out by using Genomic DNA Mini Kit (Geneaid, Korea)/ Tissue protocol according to the manufacturer's instructions. The concentration of chromosomal DNA was measured with Q5000 UV-Vis Spectrophotometer at (260nm) and DNA quality was assessed by the 260:280 nm absorbance ratio, and about 20-25 nanogram /microliter of isolated DNA aliquots were used for molecular detection of Streptococcus mutans by Nested Polymerase Chain Reaction (Nested PCR) technique according to [20- 21- 22] .

Detection of Streptococcus mutans By Nested PCR

Isolated DNA was used for detection the genus specific of Streptococci was performed by PCR depending upon pair of primers specific to 16s rDNA of the genus Streptococci in Table no.1, according to the amplification reaction program in Table no.2. And the DNA product samples of 1st amplification reaction were used as a template for the detection of Species specific S. mutans by means of 16s rDNA primer pair of Specific species S. mutans in (table no.1) according to the 2nd amplification reaction program in (table no.3) [23- 24] .

Table 1: Primer sets that used for detection of Streptococcus mutans by N PCR.

Bacterium	Sequence of Primers (5 – 3)		Length	References
Streptococci species 8UA	F	5"- AGAGTTTGATCCTGGCTCAG -3 "	1505	(23, 25)
	R	5"-TACGGGTACCTTGTTACGACTT-3 "	1492	
Streptococcus mutans	Sm1 F	5"- GGTCAGGAAAGTCTGGAGTAAAAGGCTA-3"	282	(26, 27)
	Sm2 R	5" -GCGTTAGCTCCGGCACTAAGCC-3"	282	

F= forward primer, R= reverse primer. Sm= Streptococcus mutans

Table 2: (1st Amplification reaction program) for amplifying 16S rRNA gene of the Genus Streptococcus by PCR technique according to (23,25).

No. of cycles	Stage	Temperature °C	Time
1	Initial denaturation	95	5 min.
30	Denaturation	95	30 Sec.
	Annealing	58	30 Sec.
	Elongation	72	1 min.
1	Final extension	72	5 min.

Table 3: (2nd Amplification reaction program) for amplifying 16S rRNA gene of Streptococcus mutans by Nested PCR technique according to (26-27 -38-39- 40-41) .

No. of cycles	Stage	Temperature °C	Time
1	Initial denaturation	95	5 min.
30	Denaturation	95	1 min
	Annealing	56	1 min
	Elongation	72	1.5 min.
1	Final extension	72	10 min.

DNA Analysis and Electrophoresis

The amplified DNA products of the 1st and 2nd PCR (Nested PCR) reactions were analysed by electrophoresis in a concentration of (1.5) % Agarose gel (which prepared from 1.5 gm. of very pure agarose powder melted in 100 ml. of 1X TBE buffer (0.89 M Tris Base, 0.89 M Boric acid, 20 mM EDTA) (PH 8.3), separation of amplified DNA products was done by mixing 5 µl of PCR product with 2 µl of 6x loading dye, then, the mixture was loaded into the well of the gel with the using of DNA ladder with molecular weight 1K bp. Ladder and 100 bp. (Accu Bioneer/Korea) as a molecular size marker via the agarose gel at an electric current of 90 Volts for one hour through horizontal gel electrophoresis system (Sigma Chemicals Co. USA).

Statistical Analysis

The collected data were analyzed using the statistical system and Chi-Square ($\chi 2$) test, with P-value of ≤ 0.05.

Results and Discussion

Nested Polymerase chain reaction (N-PCR) technique was subjected in the current study for detecting Streptococcus mutans in CVD samples, whole genomic DNA, which was yielded directly from CVD specimens was applied to 1st reaction of N-PCR using universal primers pair specific to 16s rDNA of the genus Streptococci., then, the positive amplified DNA products (1505 bp. size) by PCR which represent Streptococcus genus, were detected in all the 1-5 CVD samples (Fig. 1). The 2nd reaction was performed by using (DNA products of the 1st N-PCR reaction as a template) with 16s rDNA primer pair specific to the species of Streptococci mutans.

Moreover, Streptococcus mutans was specified in all positive 1-5 CVD samples. (Fig. 2) that revealed the existence of the amplified PCR products (282 bp.) as applied by (23- 31) , this procedure was identical to the

investigations of a similar related studies for identifying S. mutans targeting 16S rRNA gene which was a proper manner for determining the microbial population of the biofilms in pathological specimens of CVD [18-21-22-30] , as well as divers variety of PCR and sequencing techniques, in which amplification and nucleotide characterization of 16S rRNA gene remains using primer sets targeting the sequence common in eubacterial species, were broadly performed for identifying bacterial species [32] . Moreover, universal primers for 16S rRNA gene of species specific S. mutans was depended in a broad range of similar studies including [7-20 -21] .

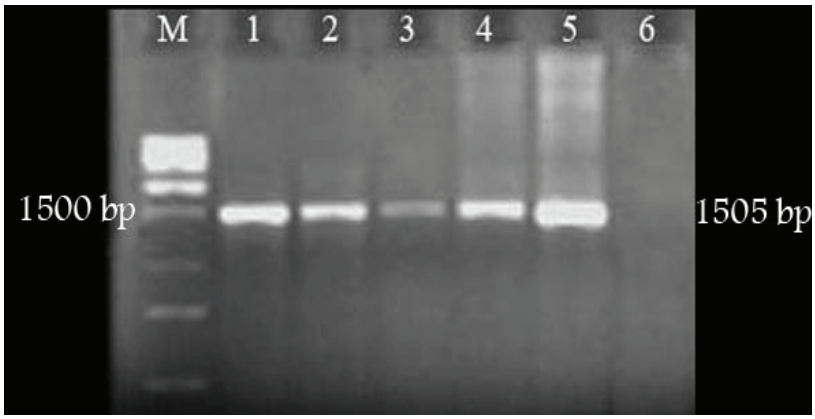

Figure 1: Agarose gel electrophoresis of DNA products of PCR amplification for 16s rDNA specific for Streptococcus genus for the biopsy samples of CVD plaques. Lane M; DNA ladder marker with molecular weight (1000 bp.), (Lanes 1, 2, 3, 4 and 5: represents positive biopsy samples of CVD patients, and Lane 6: negative sample.

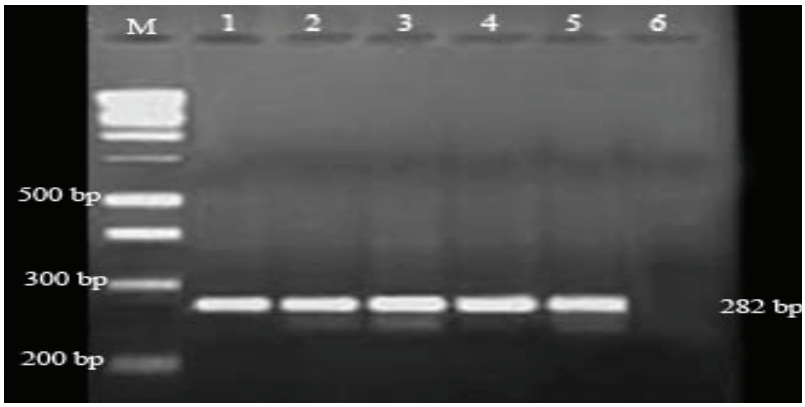

Figure 2: Agarose gel electrophoresis of DNA products of N-PCR amplification for the species specific Streptococcus mutans for the biopsy samples of CVD plaques. Lane M; DNA ladder marker with molecular weight (100 bp.), Lanes 1, 2, 3, 4, 5: positive biopsy samples of CVD patients and Lane 6: negative sample.

In the present study, specification of DNA samples of S. mutans within the biopsies of heart valve, blood vessels and atheromatic plaques of Iraqi CVD patients is the propel evidence for contributing the fundamental cariogenic pathogen in the target biofilms, DNA aliquots of S. mutans was found in 60/86(69.7%) of the enrolled CVD patients with significant importance X2= 5.882 (P-value of ≤ 0.05) in male patients were 42/60 (70 %) more than in females 18/60 (30%) table 4., this result was consistent with numerous previous related studies revealed the most frequently microorganism found in the heart valves samples was the S. mutans (89.3%), of 42 CVD patients with a mean age of 55.6-13.8 years regarding the medical conditions that led to valve replacement surgery and was agreed with Japanese patients revealed S. mutans was higher percentage (77.8)% than the other oral and periodontal bacteria, using simple PCR method and Streptococcus mutans was commonly diagnosed (69%) in the heart valve and (74%) in atheromatous plaque of the vascular wall specimens.

Table 4. Distribution of Cariogenic Streptococcus mutans in cardiovascular disease Patients and control groups.

Streptococcus mutans in Subjects						**Statistical analysis**
Variable		**Cardiovascular disease group 86**		**Control group 23**		
		(+)No, Percentage	**(-) No, Percentage**	**(+) No, Percentage**	**(-) No, Percentage**	
Gender	Male	42/60 (70 %)	11/26 (42.3 %)	3/23 (13%)	12/23(52 %)	X2= 5.882 DF= 1 P ≤ 0.05
	Female	18/60 (30 %)	15/26 (57.7 %)	3/23(13%)	5/23(22 %)	
Age	24-60 years	33/60 (55 %)	20/26 (77 %)	2/23(8.7%)	12/23(52 %)	X2= 3.687 DF= 1 P ≤ 0.05
	> 60 years	27/60 (45 %)	6/26 (23 %)	3/23(13%)	6/23(26 %)	

Daboor, et al., (2015) revealed S. mutans is actively trained in its automatic modes of biofilm formation., the cariogenic Streptococcus mutans successfully employs dietary sucrose for the production of exopolysaccharide, which proceed as a scaffold for its biofilm, thus participating to its pathogenic activity, situational stress tolerance, and resistance of many antimicrobial agents [(5)] .

On the other hand, in the current research, specification of DNA of S. mutans in CVD patients 42/60 (70%) in males more than 18/60 (30%) in females with significant importance X2= 3.687 (P-value of ≤ 0.05), this result may be elevated in the collectively number (53) of CVD males patients more than total (33) CVD females that enrolled in this study in comparison to the control group that reflects no significant difference as demonstrated in Table 4.

As well as, CVDs are commonly occurs in human due to a diverse array of intrinsic and extrinsic factors and mechanisms particularly biofilms forming bacteria like S. mutans and related members and their products, this result concur to previous finding by [(30)] concluded that increasingly evident of a broad range of mechanisms are utilized by different members of the viridans group Streptococcai to promote thrombosis in cardiac and vascular atherosclerosis plaques and the progression of CVDs such as Infective Endocardits.

Conclusions

The present study adds considerable evidence for the involvement of oral commensal bacteria S. mutans strain in human CVDs via its ability to colonize and populate in oral environment and spread throughout blood stream to cardiovascular system forming biofilms and atherosclerotic plaques in male CVD patients more than in females and in younger ages more than old peoples, S. mutans, which shows serious impacts on the human's health. So that, more work has to be done for the sake of preventing this terrible and transferrable bacterium from invading the bloodstream and eventually the endothelial tissues of the heart and preventive techniques such as brushing twice a day, reduction in sucrose rich foods, regular mouth washing, proper flossing and brushing, essentially keeping the mouth free from existence of bacteria which causes dreadful diseases.

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Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols

were approved under the Department of Microbiology and all experiments were carried out in accordance with approved guidelines.

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